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(54) Title: METHODS FOR INCREASING CATIONIC ANTIMICROBIAL PEPTIDES

(57) Abstract

Novel methods for enhancing or inducing the endogenous production of cationic antimicrobial peptides, preferably of defensins, more preferably of α defensins and β defensins, most preferably β defensins, in cells, preferably in epithelial cells, is provided. The method involves administering to cells either *in vitro* or *in vivo*, a CAP Stimulant and comprises the following steps: providing a CAP Stimulant, administering the CAP Stimulant to the cells. The CAP Stimulant comprises the inducer and optionally though preferably an aqueous carrier. The inducer is selected from the group consisting of: a bacterial cell wall extract inducer, a purified inducer, a peptidoglycan inducer and mixtures thereof. The purified inducer is preferably isolated and purified from the bacterial cell wall extract inducer. The bacterial cell wall extract inducer is preferably an extract of a commensal bacteria, such as for example, an oral bacteria, more preferably *Fusobacterium*, most preferably *Fusobacterium nucleatum*. The CAP Stimulant, when administered to cells, preferably epithelial cells, enhances the production of the cationic antimicrobial peptides, particularly defensins. Novel methods of detection of cationic antimicrobial peptide and the mRNA encoding such peptides, in cells whether the cells are in tissue, tissue samples, or in culture are provided. The methods of detection are useful for identifying individuals who are more prone to infection via the epithelia, due to low level production of cationic antimicrobial peptides. Thus, epithelial cells are removed from a patient, preferably mucosal cells, and the cells are cultured using conventional cell culture techniques. The CAP Stimulant is administered to the cultured cells, and the cationic antimicrobial peptide produced in response to the stimulant, is detected. Alternatively, the mRNA encoding such cationic antimicrobial peptides, is detected. The invention also relates to methods of making cationic antimicrobial peptides.

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METHODS FOR INCREASING CATIONIC ANTIMICROBIAL PEPTIDES

Background of the Invention

Skin and mucosa are physical barriers to the outside environment, protecting the host from noxious intruders. These barriers are not only physical; they generate potent cationic antimicrobial peptides. These peptides, important for the innate defense of a eukaryotic host, are evolutionarily ancient, and present in a wide range of eukaryotes, including insects, amphibians, birds, and mammals. Cationic antimicrobial peptides are present in the skin, the intestines, the airway, the urinary tract, the female reproductive tract, and the mouth. Cationic antimicrobial peptides provide a rapid response, within hours, to provide an initial line of defense against a broad spectrum of microbial organisms; bacteria, fungi, and some encapsulated viruses.

Attempts have been made to produce modified cationic antimicrobial peptides having enhanced antibacterial properties, utilizing synthetic or recombinant techniques. However when administered to cells, such modified cationic antimicrobial peptides have proved to be cytotoxic.

It would be desirable to have methods of to increase the defenses available to cells, that do not employ modified cationic antimicrobial peptides.

Summary of the Invention

The present invention provides novel methods for enhancing or inducing the endogenous production of cationic antimicrobial peptides, preferably of defensins, more preferably of α

defensins and ß defensins, most preferably ß defensins, in cells, preferably in epithelial cells. The method involves administering to cells either in vitro or in vivo, a CAP Stimulant and comprises the following steps: providing a CAP Stimulant, administering the CAP Stimulant to the cells.

The CAP stimulant comprises the inducer and optionally though preferably an aqueous carrier. The inducer is selected from the group consisting of: a bacterial cell wall extract inducer, a purified inducer, a peptidoglycan inducer and mixtures thereof.

The purified inducer is preferably isolated and purified from the bacterial cell wall extract inducer. The bacterial cell wall extract inducer is an extract of preferably of a commensal bacteria, such as for example oral bacteria, more preferably *Fusobacterium, most preferably Fusobacterium nucleatum*. The CAP Stimulant, when administered to cells, preferably epithelial cells, enhances the production of the cationic antimicrobial peptides, particularly defensins.

The invention also provides novel methods of detection of cationic antimicrobial peptide and the mRNA encoding such peptides, in cells whether the cells are in tissue, tissue samples, or in culture. The methods of detection are useful not only for the study of genetic distribution such as for example single nucleotide polymorphisms, but also for identifying individuals who are more prone to infection via the epithelia, due to low level production of cationic antimicrobial peptides. Thus, epithelial cells are removed from a patient, preferably mucosal cells, and the cells are cultured using conventional cell culture techniques. The CAP Stimulant is administered to the cultured cells, and the cationic antimicrobial peptide

produced in response to the CAP stimulant, is detected. Alternatively, the mRNA encoding such cationic antimicrobial peptides, is detected. The invention also relates to methods of making cationic antimicrobial peptides.

Brief Description of the Figures

Figure 1 *is a graph showing the relative ratio of hBD-2/GAPDH and interleukin 8/GAPHD.*

Detailed Description of the Invention

The present invention provides novel methods for enhancing the production of cationic antimicrobial peptides, preferably of defensins, more preferably of β-defensins, in cells, preferably in epithelial cells. As used herein "enhancing" means that the cells may have produced or may not produced, cationic antimicrobial peptide in the absence of the CAP Stimulant. Thus when say mRNA is enhanced by the CAP Stimulant, the mRNA may be increased above basal levels; the basal level may be zero or have a positive value. Similarly, Thus when say mRNA is enhanced by the CAP Stimulant, cationic antimicrobial peptides may be increased above basal levels; the basal level may be zero or have a positive value.

The method for enhancing the production of cationic antimicrobial peptides in cells, comprises the following steps; providing a CAP Stimulant, administering the CAP Stimulant to the cells. The CAP Stimulant, when administered to tissue preferably epithelial cells, enhances the production of the cationic antimicrobial peptides, particularly defensins.

The invention is a useful tool to increase the production of cationic antimicrobial peptides which are then isolated to provide a ready source for cationic antimicrobial peptides.

The invention also provides novel methods of detection of cationic antimicrobial peptide in patients either by detecting the cationic antimicrobial peptides themselves, or by detecting the mRNA encoding such cationic antimicrobial peptides. Detection in a patient sample is useful for identifying individuals who are more predisposed to infection due to reduced or absent epithelial cationic antimicrobial peptides, such as defensins. Thus, epithelial cells are removed from a patient, preferably mucosal epithelial cells, and the cells are preferably cultured using conventional cell culture techniques. The CAP Stimulant is administered to the cultured cells, and the resulting cationic antimicrobial peptide is detected. The cationic antimicrobial peptides are detected using antibodies or by extraction and analysis of the cellular protein. Alternatively, the mRNA encoding such cationic antimicrobial peptides, is detected.

The method is useful for enhancing the production of cationic antimicrobial peptides, preferably defensin in epithelial cells, preferably mucosal cells. Such cells may be in culture or in tissue and in vivo or in vitro. Such cells are derived from vertebrate sources preferably mammalian sources, more preferably human, cattle, pig, rabbit, mouse, guinea pig or rat sources.

The Cationic Antimicrobial Peptides

The cationic antimicrobial peptides are preferably β sheet, or loop structure or extended helical cationic antimicrobial

peptides. Preferably the cationic antimicrobial peptide is not a cathelin associated cationic antimicrobial peptide.

The amino acid sequence of the α defensins and the β defensins are as follows:

hBD-1 YNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK

hBD-2 VTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP

The CAP Stimulant

The CAP stimulant comprises the inducer and optionally though preferably an aqueous carrier, such as for example, phosphate buffered saline, pharmaceutically acceptable carriers and distilled water.

The inducer is selected from the group consisting of: a bacterial cell wall extract inducer, a purified inducer, a peptidoglycan inducer and mixtures thereof.

It is preferred that the CAP stimulant be free of lippopolysaccharide; by "free" it is meant below 5%. It is preferred that the CAP stimulant be free of bacterial cell wall protein; by "free" it is meant below 5%.

The Bacterial Cell Wall Extract Inducer and the Purified Inducer

The bacterial cell wall extract inducer is preferably isolated from the bacterial cell wall of a commensal bacteria, such as for example, oral bacteria, more preferably *Fusobacterium*, such as for example *F. nucleatum*, *F. russi*, most preferably *Fusobacterium nucleatum*. Other bacterial sources are *F. necrophrum*, *F. gonidiaformans*, and *F. mortiferum*.

The bacterial cell wall extract inducer contains three major types of components, LPS, proteins, and peptidoglycans. Both the LPS and proteins have been eliminated as molecules which stimulated the production of cationic antimicrobial peptides. The LPS was eliminated by the use of polymixin B sulfate. Polymixin B sulfate, which binds to and inactivates LPS, was added to the bacterial cell wall extract inducer which was subsequently administered to cells. The LPS did not stimulate hBD-2 mRNA expression; that is the hBD-2 mRNA was still enhanced by the Polymixin B sulfate/bacterial cell wall extract inducer. Unstimulated control culture showed no hBD-2 expression. Thus LPS is not responsible for the enhanced expression of the mRNA that encode cationic antimicrobial peptides.

Protein conformation is not heat stable; accordingly the bacterial cell wall extract inducer was boiled for 1 hour. Boiled cell wall extract inducer stimulated induction of of hBD-2 mRNA. Thus a protein is not responsible for the enhanced expression of the mRNA that encode cationic antimicrobial peptides.

The Purified Inducer

The purified inducer is a purified bacterial cell wall extract inducer. The purified inducer does not upregulate interleukin 8 as is characteristic with LPS and as characteristic of bacterial cell wall extract inducer.

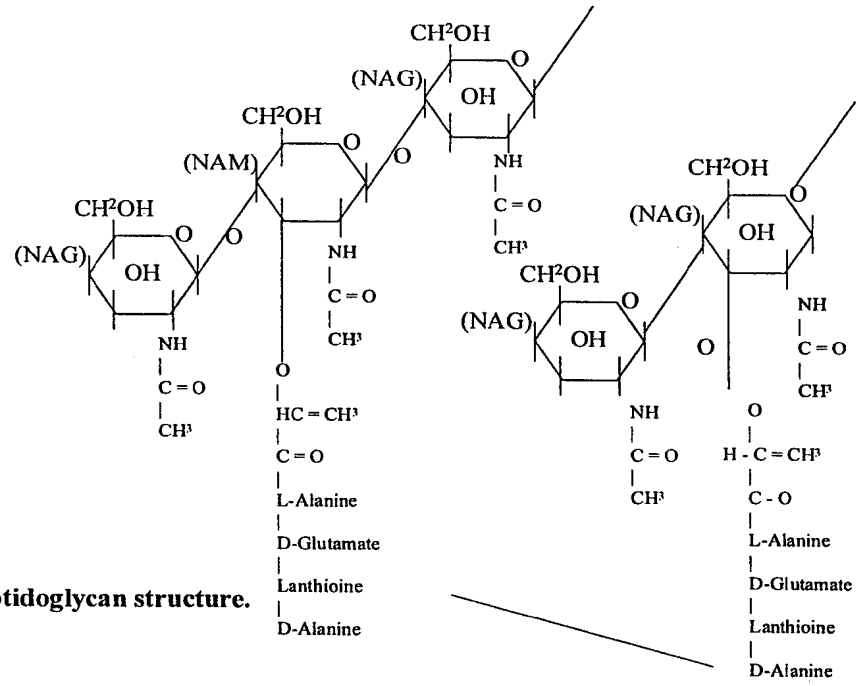
The Peptidoglycan Inducer

The preferred peptidoglycan inducer preferably comprises a at least one monsaccharide N-acetylmuramic acid bound to at least one monosaccaride N-acetyl glucosamine. Even more

preferably, the peptidoglycan polysaccharide preferably comprised of polymerized units of the monsaccharide N-acettlmuramic acid and the monosaccaride N-acetyl glucosamine. Preferably the monsaccharide N-acetylmuramic acid and the monosaccharide N-acetyl glucosamine are alternating.

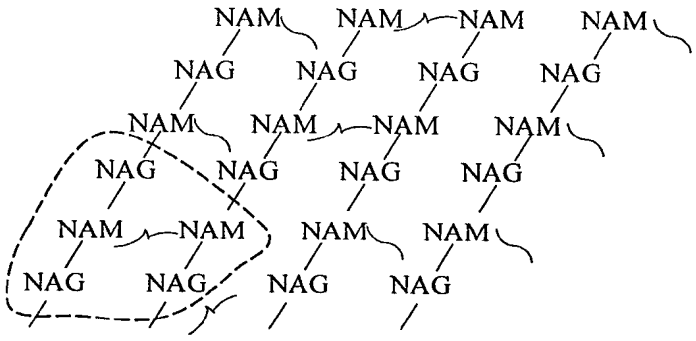
The polymer is preferably comprised of polymerized units of a muropeptide monomer having a molecular weight of preferably 800 to 2000, more preferably from about 950 to 1100, most preferably 1000 daltons.

The chain is optionally interconnected to at least one other polymer chain preferably by at least one peptide. The interconnecting peptide preferably has from about 2 to 50, more preferably 3 to 20, even more preferably from 3 to 10, most preferably 4 amino acids in length. The peptide preferably comprises the amino acid lanthionine. The structure of the most preferred peptidoglycan inducer, is shown below.



Peptidoglycan structure.

The preferred peptidoglycan inducer, as it appears in the F. nucleatum cell wall as shown below:



While the above peptidoglycan inducer is preferably isolated from bacterial cell walls as described herein, the peptidoglycan inducer may also be produced synthetically using conventional techniques.

Preparation of the CAP Stimulant

The bacterial cell wall of the bacteria is disrupted; good results have been obtained using French pressure cell disruption in phosphate buffered saline. While a range of forces will disrupt bacterial cell walls and thus are suitable, good results have been obtained using a force of 15,000 lb/in^2. The material is then centrifuged at low speed centrifugation to remove

unbroken cells; good results have been obtained using 5,000 x g for 15 minutes. The supernatant is then decanted and the supernatant is centrifuged at a higher speed centrifugation to provide a pellet; good results have been obtained using 25,000 xg, for 30 minutes to provide a bacterial cell wall extract inducer. Preferably the pellet is resuspended in phosphate buffered saline. Such procedures are described in Kennel and Holt, Oral Microbiology and Immunology, volume 15, pages 121-130, 1990). Preferably the protein content of the bacterial cell wall extract inducer is then determined using conventional techniques.

The bacterial cell wall extract inducer is used to enhance the production of cationic antimicrobial peptides in cells either in vivo or in-vitro. Good results have been obtained with bacterial cell wall extract inducer using a protein concentration of 10 µg/ml. However, the bacterial cell wall extract inducer is preferably further purified to provide the purified inducer.

The bacterial cell wall extracts are lyophilized to dryness, suspended in an aqueous solution preferably in a sodium lauryl sarkosinate aqueous solution. The suspension is then centrifuged; good results have been obtained at 100,000 x g., 4°C, for 90 minutes. The precipitate is re-suspended in 2% lithium dodecyl sulfate, dissolved in 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-lithium hydroxide (HEPES-LiOH) buffer, and centrifuged; good results have been obtained at 100,000 x g, at 4°C, for 90 minutes. The precipitate is then suspended in 4% LDS in HEPES-LiOH, boiled, then centrifuged; good results have been obtained at 100,000 x g, at 4°C, for 90 minutes. The precipitate is washed,

preferably with distilled water. The material is solvated, preferably in phosphate buffered saline; good results have been obtained using ultrasonication at a power setting of 3 watts for 5 minutes, and protein content determined.

Administration of the CAP Stimulant

The CAP Stimulant is then administered to cells either in vivo or in-vitro. The cells are incubated with the CAP Stimulant for time sufficient for the antimicrobial peptides to be produced; good results have been obtained by incubating for 24 hours at 37°C and 5% CO_2.

Where the CAP Stimulant comprises the bacterial cell extract inducer, it is preferred that preferably at least 0.5 µg/ml of the CAP Stimulant is administered to the cells. Preferably the bacterial cell wall extract inducer is administered from 1 µg/ml to 1000 µg/ml, preferably 5 µg/ml to 200 µg/ml. Where the CAP Stimulant is the purified inducer, preferably at least 0.5 µg/ml of the purified inducer, is administered to the cells. Preferably the purified inducer is administered from 1 µg/ml to 1000 µg/ml, preferably 5 µg/ml to 200 µg/ml. The CAP stimulant is added preferably in liquid form to cell cultures, and tissue samples.

The CAP stimulant is also administered topically in-vivo, preferably topically, such as for example in toothpaste, mouthwash, skin creams, and cosmetics, and to devices that come in contact with such epithelial surfaces. Such devices are for example implants, devices used in guided tissue membrane procedures, prosthesis and prosthesis adhesives, catheters orthodontic appliances, dentures, and night guards. The cationic antimicrobial peptide, particularly when applied topically is

useful to prevent infection or fight existing infections on epithelial tissues particularly mucosal surfaces.

After incubation with the CAP Stimulant, the cells are collected and preferably the mRNA is extracted. The mRNA is extracted from cells using conventional techniques. For example such extraction is preferably accomplished by lysing the cells, homogenizing the cells and extracting RNA with Rnaqueous ® (Ambion) according to manufacturer's directions. The yield of RNA is preferably measured by UV absorbance. The expected yield of RNA from keratinocytes is approximately 10 to 15 ug/10^6 cells. Preferably, the RNA is stored under refrigeration. Preferably any genomic DNA in the RNA sample is degraded prior to reverse transcription.

Complementary DNA (cDNA) is prepared from total RNA by viral reverse transcriptase enzyme using conventional techniques. The original RNA is then degraded by *E. coli* RNAse H. Next, the cDNA is amplified by polymerase chain reaction (RT-PCR) analysis. The PCR amplification is preferably accomplished using Taq polymerase, dNTPs, buffer, and specific oligonucleotide primers for hBD-2. The sequences of the hBD-2 cationic antimicrobial polypeptide are known in the art.
Intron spanning primers specific for the conserved prepro-sequences and the conserved C-terminal region are used. Products are preferably analyzed by electrophoresis in comparison with a standard DNA size ladder. DNA bands are visualized such as with ethidium bromide under UV illumination. The approximate size of the defensin antimicrobial peptide is 36 to 44 amino acids or 4000 to 6000 daltons.

Alternatively, instead of detecting mRNA encoding the cationic antimicrobial peptides, the cationic antimicrobial

peptides themselves are detected. The cationic antimicrobial peptides are extracted from cell lysates using conventional techniques such as for example by acetic acid extraction. The cationic antimicrobial peptides are then detected using conventional techniques such as by using antibodies specific for the antimicrobial peptide.

Individuals differ in their expression of β-defensins. This invention is useful to determine an individual's potential use of this type of innate host defense mechanism. It can be used in determining the cause of the problem for those who do not respond.

Examples

Preparation of the CAP Stimulant

Example A Preparation of the Bacterial Cell Wall Extract Inducer

Fusobacterium nucleatum having ATCC accesion number 25586, was grown anaerobically in 5 liters mycoplasma broth base with hemine and menadione for 36 hours - two days and harvested by centrifugation at 7000 xg for 30 minutes. The cells were washed twice with phosphate buffered saline at pH 7.2. After harvesting, *F. nucleatum* bacterial cell wall extract inducer was prepared by French pressure cell disruption in phosphate buffered saline ("PBS") (pH 7.2) at 15,000 lb/in^2. The bacterial cell walls were recovered from the supernatant by low speed centrifugation for 5,000 xg, for 15 minutes followed by high speed centrifugation for 25,000 xg, for 30 minutes according to techniques as described in Kennel and Holt, 1990). Alternatively, low speed centrifugation at 2,200 x g, for 10 minutes, at 4°C followed by high speed centrifugation at 30,000 x g, for 20 minutes. 4°C. The extract was resuspended in 0.5

ml of PBS for total protein determinations. The protein content of the bacterial cell wall extract inducer was then determined to be 5 g/ml.

Example B Preparation of the Purified Inducer

The bacterial cell wall extract inducer was produced as in example A and then the bacterial cell wall extract inducers from 5 liters of growth were lyophilized to dryness. Two hundred mg (dry wt) was suspended in 20 ml of 0.5% sodium lauryl sarkosinate aqueous solution from Sigma Chemical Co., St. Louis, MO, and the suspension was gently stirred at room temperature, for 30 minutes using a magnetic stirrer to separate out the insoluble outer membrane. The suspension was then centrifuged at 100,000 x g., 4°C, for 90 minutes. The precipitate was then suspended in 10 ml of 2% lithium dodecyl sulfate from Sigma, dissolved in 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma)-lithium hydroxide (HEPES-LiOH) buffer, and stirred at 4°C, 15 minutes, and centrifuged at 100,000 x g, at 4°C, for 90 minutes. The precipitate was then suspended in 10 ml of 4% LDS in HEPES-LiOH, boiled for one hour, then centrifuged at 100,000 x g, at 4°C, for 90 minutes. The precipitate was washed five times with distilled water. The material was then ultrasonicated using a model 60 dismembrator, from Fisher Scientific for 5 minutes to get it into solution to provide the purified inducer. The protein content was then determined.

Enhancing the Endongenous Cellular Production of Cationic Antimicrobial Peptides

Example 1 Production of Cationic Antimicrobial Peptide

The CAP Stimulant produced in example A was administered to human gingival epithelial monolayer cell cultures at 80% confluence at 10 μg/ml. The cell cultures were in serum free keratinocyte growth medium, from Clonetis Corp, San Diego, CA, which contained bovine pituitary extract, human recombinant epidermal growth factor, insulin, hydrocortisone, and gentamicin sulfate supplements according to the manufacturer's instructions. The cultures were then incubated for 24 hours at 37°C and 5% CO_2. Multiple control cultures were also run. After incubation, the enhancement of the cellular production of the cationic antimicrobial peptides was verified by comparing the mRNA levels to controls. The cells were collected and RNA extracted followed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for human β-defensin 2 (hBD-2).

The cells were homogenized by the Trizol reagent from GIBCO BRL. The RNA was extracted with Rnaqueous ® from Ambion according to the manufacturer's directions. The yield of RNA was measured by UV absorbance at 260/280 nm/ the expected yield of RNA from keratinocytes is approx. 10-15 ug/10^6 cells. The RNA was stored at -80°C.

Complementary DNA (cDNA) was prepared from the total extracted RNA by viral reverse transcriptase enzyme using the Superscript kit from Gibco BDL according to the manufacturers directions. Approximately 3 ug RNA was used per reaction with a mixture of deoxynucleotides (dNTPs), oligo d(T) primer, buffer, and enzyme. After incubation, the RNA sample was degraded by *E. coli* RNAse H, and the final mixture was made up to a final volume of 100 μl. PCR amplification was performed using Taq polymerase, dNTPs, buffer, and specific oligonucleotide primers

for hBD-2. The following primers were used to detect human β-defensin 2 hBD-2 mRNA expression:

HBD-2 (upstream)	5′	CCA GCC ATC AGC CAT GAG GGT	3′
HBD-2 (downstream)	5′	GGA GCC CTT TCT GAA TCC GCA	3′

Oligonucleotide primers for controls (housekeeping genes)

RPO[6] (downstream)	5′	ACT CTT CTT TGG CTT CAA CC	3′
RPO (upstream)	5′	AGC AGG PGT PCG ACA APG GCA	3′
Keratin 5 (upstream)	5′	GTC CTC TCC ATG GAC AAC AAC	3′
Keratin 5 (downstream)	5′	TGT CAA TCT CGG CTC TCA GCC	3′
Keratin 14 (upstream)	5′	CAG GGT GGG TGG AGA TGT CAA TG	3′
Keratin 14 (downstream)	5′	ACC ATT CCT CGG CAT CCT TGC	3′

ribosomal phosphoprotein

Intron spanning primers specific for the conserved prepro-sequences and the conserved C-terminal region were used. Temperature cycling conditions were 1 minute, 94°, 1 minute, 50°, 1-2 minute 72°. Samples were amplified for 22-35 cycles. Controls include a minus RT-template as a negative control. The control using the RNA sample without performing the reverse transcription reaction is not amplified and serves as a control for genomic DNA contamination. Products were analyzed by electrophoresis on 1% agarose gels in comparison with a standard DNA size ladder. DNA bands were visualized with ethidium bromide under UV illumination and photographed.

Evaluation of the gel revealed that the cells expressed cationic antimicrobial peptides that exhibit differential regulation by the CAP Stimulant. The size of the amplified product of DNA encoding hBD-2 is 255 base pairs. The size of the amplified of DNA encoding RPO control is 342 base pairs. All products are the predicted size. The hBD-2 is not constitutively

expressed. The hBD-2 was expressed in the cells stimulated with the CAP Stimulant. With the exception of cells receiving phorbol myristate acetate, cells that did not receive the CAP Stimulant did not express cationic antimicrobial peptides. Where the gel indicated that DNA was present as a contaminant, 10 to 12.5 µg/ml. Dnase I from GIBCO BRL was added to the remaining RNA sample that had not yet been reverse transcribed to degrade any genomic DNA present in the RNA sample.

Example 1A

The analysis of Example 1 was repeated except that the CAP Stimulant of example B was used instead of Example A.

Example 2

The process of example 1 was followed except that the following primers were used to detect the mRNA encoding hBD-1:

hBD-1 5^2-1 (upstream) 5′ ATG AGA ACT TCC TAC CTT CTG CT 3′

hBD-1 3^2-1 (downstream) 5′ TCA CTT GCA GCA CTT 3′

The hBD-1 is constitutively expressed. The size of the amplified product of DNA encoding hBD-1 is 207 base pairs. The hBD-1 mRNA was expressed in equivalent yield in the cells which received the CAP Stimulant to the cells that did not receive the CAP Stimulant.

Example 2A

The analysis of Example 2 was repeated except that the CAP Stimulant of example B was used instead of Example A.

Example 3 Detection of Cationic Antimicrobial Proteins

Cells were grown as in example 1 and the CAP Stimulant of Example A was administered as in example 1. However, the cationic antimicrobial peptides were detected rather than detecting the mRNA encoding the cationic antimicrobial peptides. Also, E. coli lippopolysaccharide, tumor necrosis factor a, P. gingivalis cell wall and PMA as a positive control, were also administered to other cell cultures to serve as controls/ and test stimulants. Specifically, the cells were harvested, as in example 1, resuspended in 5% acetic acid, and homogenized in 5% acetic acid. After overnight incubation with rotation at 4°C, the solution was centrifuged 4000 x g 4°C, and the supernatant was lyophilized. The dried acetic acid extract was resuspended in water, neutralized with NaOH to pH 7-7.5. Protein content was determined. A dot immunoblot assay was performed of serially diluted crude or CM purified fractions. Typically a 10ul drop of the sample was applied to a PVDF membrane, dried, fixed in 10% formalin in Tris buffered saline, and blocked with either 1% nonfat dried milk, or 3% gelatin in Tris buffered saline to provide a blocked sample. The blocked sample was reacted with primary anti-hBD-1 antibody, followed by reaction with an alkaline-phosphatase conjugated secondary antibody. The membrane was developed for the alkaline-phosphatase.

HBD-1 as low as 400 pg was detected. The cell lysates showed hBD-1 peptide was produced in response to the bacterial cell wall extract inducer. The oells which received the E. coli lipopolysaccharide, tumor necrosis factor a, P. gingivalis or PMA did not produce any detectable cationic antimicrobial peptide. No cationic antimicrobial peptide was produced in the lysates from HGECs, despite the fact that hBD-1 mRNA is constitutively expressed in the cells. Accordingly, the response to CAP stimulant is preferably determined by detecting

the cationic antimicrobial peptide. A western immunoblot assay confirmed the results obtained with the dot blot assay.

Example 3A

The analysis of Example 3 is repeated except that the CAP Stimulant of example B was used instead of Example A.

Example 4

The hBD-2 cationic antimicrobial peptide is detected as in Example 3, except that an anti HBD-2 antibody is used to detect the hBD-2 cationic antimicrobial peptide.

Example 4A

The analysis of Example 4 was repeated except that the CAP Stimulant of Example B was used instead of Example A.

Example 5

Cells were stimulated as in Example 1 except that hBD-2 mRNA was detected and compared with IL-8 as a positive control mRNA and with a "housekeeping" control mRNA, GAPDH. Furthermore, an RPA assay was used to quantify the enhanced expression of hBD-2 mRNA in cells in response to stimulation by increasing doses 1 μg/ml, 10 μg/ml and 100 μg/ml of the CAP Stimulant. The results are shown in Figure 1.

The results showed an increasing amount of hBD-2 mRNA with increasing doses of the CAP Stimulant. The IL-8 mRNA was not detected in unstimulated cells, but was elevated even at low doses, that is 1 μg/ml of the CAP Stimulant. HBD-2 mRNA expression is upregulated in a dose-dependent manner with CAP Stimulant. The hBD-2 mRNA is not detectable in the unstimulated control culture or in the culture stimulated with the lowest

dose that is 1 μg/ml, and increases with 10 μg/ml and 100 μg/ml doses.

Example 5A

The analysis of Example 5 was repeated except that the CAP Stimulant of Example B was used instead of Example A.

Example 6

Biopsy samples of inflamed and non-inflamed gingival tissue were collected from 15 normal people, and 12 people having periodontal disease. The hBD-1 and hBD-2 mRNA levels were evaluated using the reverse transcriptase PCR procedure described as in Example 1. Human keratin 5 (HK5) was used as an epithelial-specific control (235 bp fragment). All products were amplified for 25 or 28 cycles.

The hBD-1 is expressed in normal and inflamed tissue. The hBD-2 is more highly expressed in inflamed tissue. The levels of mRNA encoding the hBD-1 and hBD-2 in normal, non-inflamed tissue varied among individuals.

Example 7 Determining Patient lLevels of β-defensin mRNA and Peptide

A noninvasive cytobrush available from (Epicentre Technology, Madison, WI) is used to gently scrape gingival tissue and collect surface cells from patients. The contents are placed in 5% acetic acid, and the protein is extracted and detected as in example 2 or by ELISA.

Example 7A

The analysis of Example 7 was repeated except that the CAP Stimulant of example B was used instead of Example A.

Example 8 Detection in Biopsy Samples

Samples of human gingival tissue or the retromolar pad were collected from 15 greater number patients. The tissue was fixed carnoys fixative, immbeded in parraffin, sectioned and stained with an anti-hBD-1 antibody or stained with an anti-hBD-2 antibody. Specifically, the tissue was incubated overnight with antibody, washed, then incubated with biotin labeled secondary antibody and stained using peroxidase conjugated with avidin and biotin with diaminobenzidine as a coloring reagent. The samples were evaluated microscopicaly at 4 x and 20 x magnification.

Microscopic inspection revealed that hBD-1 peptide is expressed in the epithelial of gingival tissue samples. The hBD-1 peptide was detected in localized areas of the tissue. The strongest expression was seen in the region of the gingival margin, and in the upper (surface) portion of the oral epithelium. Peptide expression at the gingival margin corresponds to the area of maximum exposure to bacterial plaque which accumulates at the interface of the tooth surface and the gingiva. These results suggest localized expression of the hBD-1 peptide even though the mRNA is constitutively expressed in suprabasal epithelial cells. The result indicates that there is post-transcriptional regulation of hBD-1 in response to exposure to bacteria and bacterial products.

In a patient with hereditary gingival hyperplasia, hBD-1 peptide is expressed in the gingival epithelium with the strongest reaction in the upper half of the epithelium and at the gingival margin and sulcus (hBD-1 monoclonal antibody).

These regions are those which are most exposed to bacterial plaque. The epithelium is clearly delineated with the anti-keratin antibody (AE3).

The hBD-2 peptide was also detected in gingival tissue biopsy sections in the region of the gingival margin , quite similar to the expression of hBD-1, again, corresponding to the region of maximum exposure to bacterial plaque.

Example 9

HGEC cells were grown on glass cover slips to 80% confluence and stimulated overnight with bacterial cell wall extract inducer containing the CAP Stimulant of Example A. Next the cells were fixed in 4% paraformaldehyde for 5 minutes, and permeabilized with acetone for 5 minutes at 4°C. A primary anti-hBD-2 antibody (polyclonal, 1/500) was applied overnight. The sample was incubated with FITC labeled secondary antibody was applied.

Fluoroescent microscopic examination revealed that hBD-2 expressed only in the cells which received the CAP stimulant, but not in the control cells.

Example 9A

The analysis of Example 9 is repeated except that the CAP Stimulant of example B is used instead of Example A.

Determining Abnormal expression of β-defensins and to Detect Genetic Polymorphisms

The method of stimulating epithelial cells with CAP Stimulant is useful in classifying genetic polymorphisms. The response of individuals to stimulation by the cationic

antimicrobial peptide are classified as affecting transcription, translation, or protein processing and secretion or activation. Genetic polymorphisms are determined by first collecting cells preferably oral cells preferably by a non-invasive procedure such as cytobrush collection. The genomic DNA is then extracted and amplified using for example the PCR techniques described in example 1 and amplified using exon-specific primer pairs or promoter specific primer pairs. Amplified products include, for example, promoter, spliced junctions and untranslated regions in addition to coding sequences. The amplified genomic DNA is then sequenced using conventional techniques.

Stimulation of innate host defenses by commensal bacterial cell wall factors is an important factor in fighting serious infection by pathogenic organisms. Individuals differ in their expression of β-defensin mRNA. Post-translational processing is a key step for production and secretion of active peptide. The causes for abnormal expression are believed to be mutations or genetic polymophisms that lead to altered mRNA in response to stimulation. This includes effects on the amount of mRNA expression, amount of mature peptide expression from precursor protein, and function of the peptide.

Cells which exhibit an enhanced level of mRNA encoding a cationic antimicrobial peptides comparable to control cells and display enhanced cationic antimicrobial peptides would be classified as normal. Cells which exhibit an enhanced level mRNA encoding a cationic antimicrobial peptides but do not exhibit a concomitant increase cationic antimicrobial peptides would be classified as having a post-transcriptional defect. Cells which do not exhibit enhanced level of mRNA encoding a cationic antimicrobial peptides or only a low response and do not exhibit increased cationic antimicrobial peptides would be

classified as having either a promoter defect or a nonsense mutation in the coding region, or a genetic deletion.

Materials and Methods

Antibodies to cationic antimicrobial peptides are prepared using conventional techniques. The cationic antimicrobial peptides used to produce such antibodies are made using conventional techniques. For example, recombinant hBD-1 and hBD-2 are generated using an insect cell/baculovirus protein expression system as described in Porter EM, E vanDam, EV Valore, T Ganz. 1997, Broad-spectrum antimicrobial activity of human intestinal defensin 5. *Infect. Immu.* 65:2396-2401; and Valore EV, E Martin, S Harwig, T Ganz. 1996, Intramolecular inhibition of human defensins HNP-1 by its propiece. *J. Clin. Invest.* 97:1624-29.

Gingival Epithelial Cell Cultures

Normal oral gingival tissue, from explants over impacted third molars, is incubated overnight with dispase. The epithelium is gently lifted off, pipetted to loosen cells, and placed in a T75 flask for culture in KGM medium as described in and Watson, 1990, Cell Dev. Biol. volume 26, page 589-595, which is incorporated herein by reference. Epithelial cells are expanded in culture (2 passages) then frozen in vials of 10^5 cells. On vial is then expanded into 5 T75 flasks. After one week these are plated in T150 flasks for further expansion is needed, or used to seed at a density of approx. 3,000 cells/cm^2 or 10^4/well in a 12-well culture dish. The human gingival epithelial cells are grown under standard conditions in serum

free medium until confluent. The media was changed twice weekly.

Ribonuclease Protection Assay (RPA)

The RPA assay measures a mRNA encoding cationic antimicrobial peptides by hybridization with a labled antisense probe. Specifically antisense RNA for cationic antimicrobial peptides, typically the hBD-2 and antisense RNA for the controls were prepared using conventional techniques. The probes were labeled with psoralen-biotin reagent, from Ambion BrightStar according to the manufacturere's instructions. The psoralen-biotin reagent is a non-isotopic method of labeling the probe. Each probe has a distinct size of the protected fragment for convenience in the assay; hBD-2, 195 base pairs (bp); IL-8, 620 bp; and GAPDH control, 316 bp. The probes were incubated with mRNA extracts for 18 hours at 42 at °C with at least 4 fold excess of probes Single stranded mRNA which have not hybridized to the probe as well as units of the non-hybridized probe are enzymatically digested with of mixture of 0.0375 kunitz units of ribonuclease A, 1.5 and units of ribonuclease T-1, from Ambion acc to there directions for 30 minutes at 37°C. The probe - hybridized mRNA complexes are then separated by electrophoresis, transferred to a nitrocellulose membrane, and the hybridized probe was detected by conventional chemiluminescent methods and quantitated by densitometry, using Kodak id gel analysis software.

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What is claimed is :

1. A method of enhancing the production of cationic antimicrobial peptides in epithelial cells, comprising the following steps:

providing a CAP Stimulant;

administering the CAP Stimulant to the cells.

2. The method of claim 1, wherein the CAP Stimulant comprises an inducer selected from the group consisting of a bacterial cell wall extract inducer, a purified inducer, a peptidoglycan induce and mixtures thereof.

3. The method of claim 2, wherein the CAP Stimulant further comprises an aqueous carrier.

4. The method of claim 1, wherein the cationic antimicrobial peptides is a defensin.

5. The method of claim 1, wherein the cationic antimicrobial peptides is β-defensin.

6. The method of claim 1, where in the bacterial cell wall extract inducer is extracted from *Fusobacterium*.

7. The method of claim 1, wherein the inducer is the bacterial cell wall extract inducer, which is extracted from *Fusobacterium nucleatum; and the* antimicrobial peptide is defensin.

8. The method of claim 1, wherein the inducer is the purified inducer, purified from bacterial cell wall extract inducer which is extracted from *Fusobacterium nucleatum; and the* antimicrobial peptide is defensin.

9. The method of claim 1, wherein the inducer is the peptidoglycan inducer; and the antimicrobial peptide is defensin.

10. The method of claim 9, wherein the peptidoglycan inducer comprises:
at least one N-acetylmuramic acid bound to at least one N-acetyl glucosamine;
and a peptide attached to the either the N-acetylmuramic acid or the N-acetyl glucosamine having 2 to 50 amino acids;
wherein at least one of the amino acids is lanthionine.

11. The method of claim 1, wherein the cells are within tissue located in a vertebrate.

12. The method of claim 1, wherein the cells are in cell culture.

13. The method of claim 11, wherein the CAP stimulant is administered to said cells in tissue by applying to a device which is then placed on the tissue.

14. A method of detecting cellular cationic antimicrobial peptide comprising the following steps:

a. providing a epithelial cells;

b. administering a CAP Stimulant to the cells; and

c. detecting the cationic antimicrobial peptide or the mRNA encoding the cationic antimicrobial peptide.

15. The method of claim 14, further comprising the step of:
administering the anti-cationic antimicrobial peptide antibody to the cells;
wherein the cationic antimicrobial peptide located within the cell or on surface of cell is detected by anti-cationic antimicrobial peptide antibody.

16. The method of claim 14, further comprising the steps of:
after step b., isolating the cellular mRNA;
after step c., quantifying the isolated mRNA.

17. The method of claim 14, wherein individual with reduced cationic antimicrobial peptide production is identified.

18. The method of claim 14, wherein the cells are human gingival epithelial cells.

19. The method of claim 14, wherein the cationic antimicrobial peptide is a defensin.

20. The method of claim 14, cationic antimicrobial peptide is β-defensin.

21. A method of making cationic antimicrobial peptide comprising the following steps:
providing epithelial cells;
providing a CAP Stimulant;
administering the CAP Stimulant to the cells; and

extracting the cationic antimicrobial peptide from the cells.

22. The method of claim 21, wherein the cationic antimicrobial peptide is a defensin.

23. The method of claim 21, cationic antimicrobial peptide is β-defensin.

24. A method for enhancing the production of cationic antimicrobial peptide in epithelial cells, comprising the following steps:

providing a bacterial cell wall extract inducer;

administering said bacterial cell wall extract inducer to the cells.

25. The method of claim 24, wherein the cationic antimicrobial peptide is a defensin and the bacterial cell wall extract inducer is extracted from *Fusobacterium nucleatum*.

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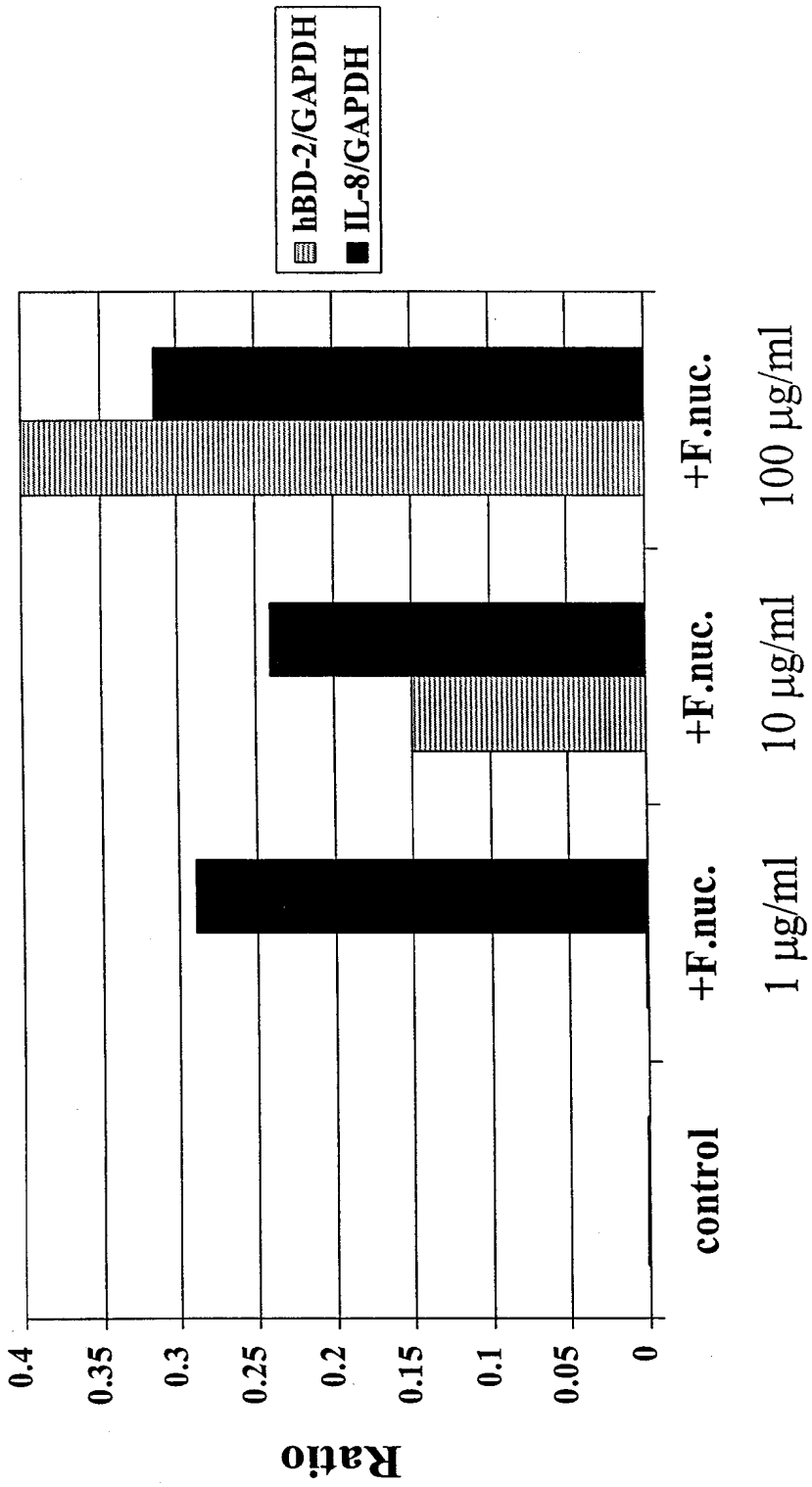
Figure 1


hBD-2/GAPDH
IL-8/GAPDH
Ratio
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
control
+F.nuc.
1 µg/ml
+F.nuc.
10 µg/ml
+F.nuc.
100 µg/ml